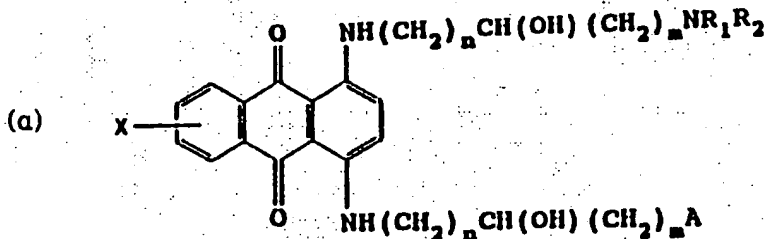




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/US91/08026</p> <p>(22) International Filing Date: 30 October 1991 (30.10.91)</p> <p>(30) Priority data: 609,179 2 November 1990 (02.11.90) US 609,252 5 November 1991 (05.11.91) US</p> <p>(60) Parent Application or Grant (63) Related by Continuation US 609,252 (CIP) Filed on 5 November 1990 (05.11.90)</p> <p>(71) Applicant (for all designated States except US): SPINX PHARMACEUTICALS CORPORATION [US/US]; Two University Place, Durham, NC 27717 (US).</p>		<p>(72) Inventors; and (75) Inventors/Applicants (for US only): JIANG, Jack, B. [US/US]; 1604 Skye Drive, Chapel Hill, NC 27516 (US). JOHNSON, Mary, George [US/US]; 1601 North Greensboro Street, Carrboro, NC 27510 (US).</p> <p>(74) Agents: CALDWELL, John, W. et al.; Woodcock Washburn Kurtz Mackiewicz &amp; Norris, One Liberty Place, 46th Floor, Philadelphia, PA 19103 (US).</p> <p>(81) Designated States: AT (European patent), AU, BE (European patent), BR, CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent), US.</p> <p><b>Published</b> With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>

(54) Title: BIS-(HYDROXYALKYLAMINO)-ANTHRAQUINONE INHIBITORS OF PROTEIN KINASE C



## (57) Abstract

The present invention provides novel substituted anthraquinones having formula (a), wherein  $R_1$  and  $R_2$  are independently H,  $C_1$ - $C_{10}$  alkyl, aryl, arylalkyl, alkylaryl, or  $R_1$  and  $R_2$  taken together with the adjacent nitrogen atom N form a substituted or unsubstituted cyclic ring which may contain additional heteroatoms; n and m are independently 1, 2, or 3; A is halogen, OH, alkoxy,  $OCO(NR_3R_4)$ ,  $S-C(NH_2)=NR_5$ , or when  $m = 1$ , comprises an oxirane ring with the adjacent oxygen atom;  $R_3$ ,  $R_4$ , and  $R_5$  are independently H, alkyl, or aryl; X is H, OH,  $NR_6R_7$ , Cl, Br, I, F, alkyl, aryl, alkoxy, aryloxy,  $COOR_8$ , or  $CONR_9R_{10}$ ; and  $R_6$ ,  $R_7$ ,  $R_8$ ,  $R_9$ , and  $R_{10}$  are independently H, lower alkyl, or aryl useful for inhibiting protein kinase C and treating conditions related to, or affected by inhibition of protein kinase C, particularly cancer tumors, inflammatory disease, reperfusion injury, and cardiac dysfunctions related to reperfusion injury.

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**BIS-(HYDROXYALKYLAMINO)-ANTHRAQUINONE  
INHIBITORS OF PROTEIN KINASE C**

**Field of the Invention**

The present invention relates to the field of  
5 treatments for inflammatory and cardiovascular diseases,  
and chemotherapeutic agents. More particularly, the  
present invention relates to novel asymmetric 1,4-bis-  
(amino-hydroxylalkylamino)-anthraquinones for inhibiting  
the enzyme protein kinase C.

**10 Reference to Related Applications**

The present application is a continuation-in-  
part application of U.S. Application Serial no. 07/609,252,  
filed November 5, 1990 in the names of Jack B. Biang and  
Mary George Johnson entitled "BIS-(HYDROXYALKYLAMINO)-  
15 ANTHRAQUINONE INHIBITORS OF PROTEIN KINASE C", which is in  
turn a continuation in part application of U.S. Application  
Serial no. 07/609,179, filed November 2, 1990, the  
disclosures of both of which are hereby incorporated by  
reference as if fully set forth herein.

**20 Background of the Invention**

Protein kinase C (PKC) is a family of calcium  
stimulatable and phospholipid-dependent serine/threonine-  
specific protein kinases which play an important role in  
cellular growth control, regulation, and differentiation.  
25 protein kinase C is also fundamental to the processes

involved in tumorigenicity, since it is the major high-affinity receptor for several classes tumor promoters as well as for endogenous cellular diacylglycerols. These tumor promoters also stimulate protein kinase C catalysis.

5 Castagna et al. (1982) J. Biol. Chem. 257: 7847 reported direct activation of protein kinase C by tumor-promoting phorbol esters. The mechanisms of protein kinase C action have been described in U.S. Patent 4,816,450 issued March 28, 1989 to Bell et al., the disclosures of which are  
10 specifically incorporated as if fully set forth herein. protein kinase C is activated by diacylglycerol (DAG), a neutral lipid, and when activated will transfer the  $\gamma$ -phosphate of MgATP to a serine or threonine residue on a substrate protein.

15 Since the activation of protein kinase C have been implicated in several human disease processes, including cancer tumors, inflammation, and reperfusion injury, inhibition of protein kinase C should be of great therapeutic value in treating these conditions.

20 Protein kinase C inhibitors have been reported to potentiate the antitumor activity of cis-platin both in vitro and in vivo (Grunicke et al. (1989) Adv. Enzyme Regul. 28: 201; and German Offenlegungsschrift DE 3827974). In addition, it has been suggested that protein kinase C  
25 would be a potential target for therapeutic design because of its central role in cell growth (Tritton, T.R. and Hickman, J.A. Cancer Cells 2: 95-102 (1990)).

Protein kinase C inhibitors have been demonstrated to block platelet aggregation and release of  
30 neutrophil activating agents such as platelet activating factor (PAF) (Schachtele et al. (1988) Biochem. Biophys. Res. Commun. 151: 542; Hannun et al. (1987) J. Biol. Chem. 262: 13620; Yamada et al. (1988) Biochem. Pharmacol. 37: 1161). protein kinase C inhibitors have also been shown to inhibit  
35 neutrophil activation, and chemotactic migration (McIntyre et al. (1987) J. Biol Chem. 262: 15730; Lambreth et al. (1988) J. Biol. Chem. 263: 3818; Pittet et al. (1987) J.

Biol. Chem. 262: 10072; and Gaudry et al. (1988) Immunology 63: 715), as well as neutrophil degranulation and release of proteolytic enzymes and reactive oxygen intermediates (Wilson et al. (1986) J. Biol. Chem. 261: 12616; Fujita et al. (1986) Biochem. Pharmacol. 35: 4555; Berkow et al. (1987) J. Leukoc., Biol. 41: 441; Salzer et al. (1987) Biochem. Biophys. Res. Commun. 148: 747; Kramer et al. (1989) J. Biol. Chem. 262: 5876; and Dewald et al. (1989) Biochem. J. 264: 879). Thus inhibitors of protein kinase C have the capability of blocking all three of the most significant mechanisms of pathogenesis associated with myocardial reperfusion injury, and should thus have a decided therapeutic advantage. Additionally, the inhibitory effect of protein kinase C inhibitors on keratinocytes, and on the oxidative burst in neutrophils will lead to an anti-inflammatory effect.

Substituted anthraquinones have been reported for various uses, including cancer treatment. U.S. Patent 3,960,751 issued June 1, 1976 to Moriyama et al. discloses substituted anthraquinones useful as pleochroic dyes. U.S. Patent 4,598,155 issued July 1, 1986 to Adam discloses tetrazole substituted anthraquinones useful as dyes.

U.S. Patent 4,762,648 issued August 9, 1988 to Stache et al. discloses mono-functional and bis-functional anthraquinone-(oxy-2,3-oxidopropanes) useful as intermediates in the preparation of drugs possessing  $\beta$ -receptor blocker action and as crosslinking agents in the preparation of polymers. The compounds also exhibit cytostatic activity.

Several patents disclose the use of substituted anthraquinones for treatment of neoplasms. U.S. Patent 4,894,451 issued January 16, 1990 to Krapcho et al. discloses unsymmetrical 1,4-bis-(aminoalkylamino)-anthracene-9,10-diones useful in the treatment of neoplasms. U.S. Patent 4,310,666 issued January 12, 1982 to Zee-Cheng et al. discloses 1,4-bis-(substituted aminoalkylamino)-anthraquinones useful in the treatment of

neoplasms. U.S. Patent 4,526,989 issued July 2, 1985 to Murdock et al discloses symmetrical 1,4-bis(substituted-amino)-5,8-dihydroxyanthraquinones useful as chelating agents and for inhibiting the growth of tumors. U.S. Patent 4,197,249 issued April 8, 1980 to Murdock et al also discloses symmetrical 1,4-bis(substituted-amino)-5,8-dihydroxyanthraquinones useful as chelating agents and for inhibiting the growth of tumors. U.S. Patent 4,540,788 issued September 10, 1985 to Murdock discloses 1,4-bis[ - aminoalkyl)amino]-9,10-anthracenediones and leuco bases thereof which are useful as chelating agents and for inducing regression of leukemia and/or inhibition of tumor growth in mammals. Japanese patent 19819 issued May 7, 1990 discloses substituted anthraquinones having substituents at positions 5 and 6 of the anthraquinone ring structure which are useful as anti-tumor agents alone or in combination with other anti-tumor agents.

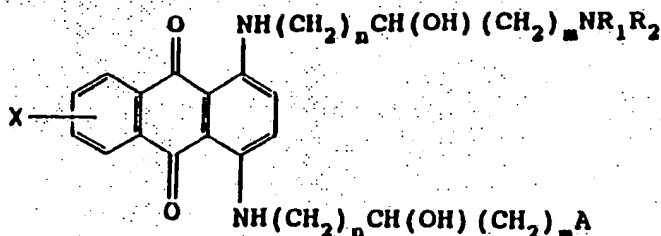
German Offenlegungsschrift DE 3827974 A1 discloses therapeutic preparations comprising a protein kinase C inhibitor in combination with a lipid, a lipid analogue, a cytostatic agent or phospholipase inhibitor useful for cancer therapy. However, none of the protein kinase c inhibitors disclosed in this publication are substituted anthraquinones.

Although substituted anthraquinones have been reported for cancer treatments, substituted anthraquinones such as Mitoxantrone, are known to be associated with side effects, mainly immunosuppressive activity, and drug resistance. Accordingly novel cancer treatments that avoid some or all of these drawbacks are needed.

Further, inflammation and reperfusion injury, particularly pertaining to cardiac injury, are common conditions for which there exists no definitive treatment despite extensive research and appropriate treatments for these conditions are needed.

### Summary of the Invention

The present invention provides novel asymmetric substituted anthraquinones having the formula



wherein  $R_1$  and  $R_2$  are independently H,  $C_1$ - $C_{10}$  alkyl, aryl,  
 5 arylalkyl, alkylaryl, or  $R_1$  and  $R_2$  taken together with the  
 adjacent nitrogen atom N form a substituted or  
 unsubstituted cyclic ring, which ring may optionally  
 contain additional heteroatoms including oxygen, nitrogen  
 or sulphur; n and m are independently 1, 2, or 3; A is  
 10 Halogen, OH, alkoxy,  $\text{OCO}(\text{NR}_3\text{R}_4)$ ,  $\text{S}-\text{C}(\text{NH}_2)=\text{NR}_5$ , or when m =  
 1, comprises an oxirane ring with the adjacent oxygen atom;  
 $R_3$ ,  $R_4$ , and  $R_5$  are independently H, alkyl, or aryl; X is H,  
 OH,  $\text{NR}_6\text{R}_7$ , Cl, Br, I, F, alkyl, aryl, alkoxy, aryloxy,  $\text{COOR}_8$ ,  
 or  $\text{CONR}_9\text{R}_{10}$ ; and  $R_6$ ,  $R_7$ ,  $R_8$ ,  $R_9$ , and  $R_{10}$  are independently H,  
 15 lower alkyl, or aryl.

The novel compounds of the invention are useful  
 for inhibiting protein kinase C and are further useful for  
 treating conditions related to, or affected by inhibition  
 of protein kinase C, particularly cancer tumors,  
 20 inflammatory disease, myocardial reperfusion injury, and  
 cardiac dysfunctions related to reperfusion injury.  
 Inhibition of protein kinase C can lead to inhibition of  
 growth of cells and can thereby produce an anti-tumor  
 effect. Further, inhibition of protein kinase C can also  
 25 lead to inhibition of the oxidative burst in neutrophils,  
 platelet aggregation, and keratinocyte proliferation,

whereby an anti-inflammatory effect is achieved. The inhibitory activities of the compounds of the invention against platelet aggregation, neutrophil activation, and neutrophil release demonstrate their usefulness in treating  
5 reperfusion injury, particularly myocardial reperfusion injury.

The compounds of the invention are expected to be particularly useful in the treatment of tumors resistant to treatment with other chemotherapeutic agents.

10 Surprisingly, compounds of the invention are able to inhibit protein kinase C even in adriamycin or Mitoxantrone resistant cells, and thus are not cross-resistant with these chemotherapy agents.

Compounds of the invention are able to inhibit  
15 proliferation of tumor cells at low concentrations (less than 1  $\mu$ M) which should lessen then potential for deleterious side effects of compounds of the invention when administered for treatment of tumors.

The compounds of the invention appear to have no  
20 effect on cAMP dependent protein kinase C and should consequently have no effect on the metabolic pathways associated with stimulation of protein kinase C by cAMP.

This invention is more particularly pointed out in the appended claims and is described in its preferred  
25 embodiments in the following description.

#### Brief Description of the Drawings

Figure 1 shows the synthetic route of Scheme I which is used for synthesis of compounds of the invention.

Figure 2 shows the synthetic route of Scheme II  
30 which is used for synthesis of compounds of the invention.

Figure 3 shows the synthetic route of Scheme III which is used for synthesis of compounds of the invention.

Figure 4 shows a graphical representation of the median MCF-7 tumor weight of control and mice treated with  
35 compound 1b-1 vs. days post implant of the tumor. Tumor

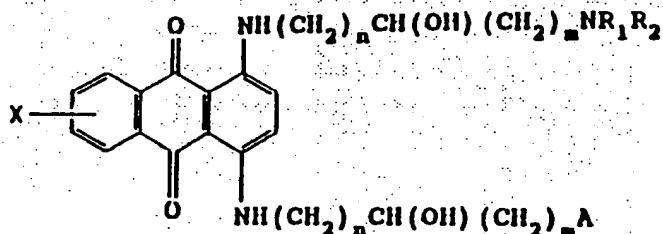


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size for control mice is shown by solid circles; tumor weight for test mice is shown by solid squares.

### Detailed Description of the Invention

The present invention provides novel substituted  
5 anthraquinones having Formula I



I

- $R_1$  and  $R_2$  are preferably independently H,  $C_1$ - $C_{10}$  alkyl, aryl, arylalkyl, alkylaryl, or  $R_1$  and  $R_2$  taken together with the adjacent nitrogen atom N form a
- 10 substituted or unsubstituted cyclic ring; more preferably H or lower alkyl; most preferably lower alkyl.  $m$  and  $n$  are preferably independently 1, 2, or 3; more preferably 1 or 2; most preferably 1. A is preferably halogen, OH, alkoxy,  $\text{OCO}(\text{NR}_3\text{R}_4)$ ,  $\text{S-C}(\text{NH}_2)=\text{NR}_5$ , or when  $m = 1$ , comprises an
- 15 oxirane ring with the adjacent oxygen atom; more preferably halogen,  $\text{OCO}(\text{NR}_3\text{R}_4)$ ,  $\text{SC}(\text{NH}_2)=\text{NR}_5$ , or when  $m = 1$ , comprises an oxirane ring with the adjacent oxygen atom; most preferably Cl, Br,  $\text{OCO}(\text{NR}_3\text{R}_4)$ ,  $\text{SC}(\text{NH}_2)=\text{NR}_5$ , or when  $m = 1$ , comprises an oxirane ring with the adjacent oxygen atom.
- 20 Halogen as used herein includes fluorine, chlorine, bromine, and iodine.  $R_3$ ,  $R_4$ , and  $R_5$  are preferably independently H, alkyl, or aryl; more preferably H or alkyl; most preferably H. X is preferably H, OH,  $\text{NR}_6\text{R}_7$ , Cl, Br, I, F, alkyl, aryl, alkoxy, aryloxy,  $\text{COOR}_8$ , or  $\text{CONR}_9\text{R}_{10}$ ;
- 25 more preferably H, HO, Cl, Br, I, F, or alkoxy; most preferably H or OH.  $R_6$ ,  $R_7$ ,  $R_8$ ,  $R_9$ , and  $R_{10}$  are independently H, lower alkyl, or aryl. As used herein, the term lower alkyl is intended to mean  $C_1$ - $C_6$  alkyl.

As used herein, alkyl substituents include straight chain, branched and cyclic moieties, preferably straight chain species. While saturated species are preferred, unsaturated sites may find utility in some substituents. Alkaryl substituents are preferably substituted benzene moieties, with ortho, meta, and para substituents each believed to be useful. Multiple substitutions are similarly useful. Aalkyl substituents are preferably benzene substituted alkanes including benzyl, phenylethyl, phenylpropyl, etc.

The compounds of the invention are useful for treating conditions related to, or affected by inhibition of protein kinase C, particularly cancer tumors, inflammatory disease, reperfusion injury, and cardiac dysfunctions related to reperfusion injury. Accordingly, another aspect of the invention provides methods and pharmaceutical compositions for inhibiting protein kinase C which comprise contacting protein kinase C with an inhibitory amount of a substituted anthraquinone compound of the invention. The pharmaceutical compositions of the invention comprise a substituted anthraquinone compound of the invention and a pharmaceutically acceptable carrier of diluent.

The invention also provides methods of inhibiting an oxidative burst in neutrophils which comprise contacting a neutrophil with a protein kinase C inhibitory concentration of a substituted anthraquinone compound of the invention, or contacting the neutrophil with an amount of a compound of the invention effective to inhibit such oxidative outburst.

The invention further provides methods for treating inflammation which comprise administering to a mammal suffering from inflammation a protein kinase C inhibitory concentration of a substituted anthraquinone compound of the invention, or administering to the mammal an amount of a compound of the invention effective to inhibit inflammation.

The invention additionally provides methods for inhibiting growth of mammalian tumor cells which comprises contacting a mammalian tumor cell with a protein kinase C inhibitory concentration of a substituted anthraquinone compound of the invention, or contacting the tumor cell with an amount of a compound of the invention effective to inhibit growth of the tumor cell.

An additional embodiment of the invention provides methods for treating mammalian tumors which comprise administering to a mammal having a tumor a protein kinase C inhibitory concentration of a substituted anthraquinone compound of the invention, or administering to the mammal having a tumor an amount of a compound of the invention effective to inhibit growth of the tumor.

Another embodiment of the invention provides methods of inhibiting mammalian keratinocyte proliferation which comprises administering to a mammalian keratinocyte a protein kinase C inhibitory amount of a compound of the invention, or administering to the keratinocyte an amount of a compound of the invention effective to inhibit proliferation of the keratinocyte.

An additional embodiment of the invention provides the use of a substituted anthraquinone compound of the invention in the preparation of a medicament for inhibiting protein kinase C. The compound of the invention is preferably present in the medicament in a therapeutically effective amount.

Surprisingly, the compounds of the invention are not cross-resistant to adriamycin (Doxorubicin HCl, Adria Laboratories, Dublin, Ohio, an antibiotic-derived chemotherapy agent) or Mitoxantrone (Novantrone®, Lederle Laboratories, Pearl River, New York, a synthetic anthracene dione chemotherapy agent), and remain effective in reducing growth of tumor cells resistant to these well-known chemotherapy agents. The lack of cross-resistance with adriamycin or Mitoxantrone indicates that the compounds of

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the invention will be particularly useful for treatment of adriamycin or Mitoxantrone resistant tumors.

Cancer is a disease characterized in part by uncontrolled cell growth. Protein kinase C is directly involved in cellular growth control and is believed to be involved in tumor formation. Protein kinase C is the major, if not exclusive, intracellular receptor of phorbol esters which are very potent tumor promoters. Phorbol esters and other tumor promoters bind to and activate protein kinase C. Since diacylglycerol (DAG) and phorbol esters interact at the same site, DAG's have been suggested to be the "endogenous phorbol esters" by analogy with the opiate receptor where the conservation of a high affinity receptor implied the existence of an endogenous analogue. DAG has been shown to increase the affinity of protein kinase C for  $Ca^{+2}$  and phospholipid and thus activates protein kinase C at cellular levels of these essential cofactors. Extracellular signals including hormones, growth factors, and neurotransmitters are known to stimulate phosphatidylinositol turnover resulting in the generation of  $IP_3$  and DAG. Structures of 40 distinct oncogenes of viral and cellular origin have revealed that oncogenes encode altered forms of normal cellular proteins. Several of the gene products appear related to growth factors or other elements involved in transmembrane signalling. These oncogene products appear to function by altering the level of critical second messengers. Cells transformed with the oncogenes ras, sis, erbB, abl, and src have been shown to contain elevated levels of DAG which is then believed to activate protein kinase C. Indeed studies on ras transformed cells have shown protein kinase C activation to concomitant with elevation of DAG.

Phorbol esters, such as phorbol myristate acetate (PMA), have complex effects on cells including effects on membrane function, mitogenesis, differentiation, and gene expression. Synthetic diacylglycerols mimic many of the effects of PMA in vitro and inhibitors of protein kinase C

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have been shown to block PMA-induced effects on cells. Thus, protein kinase C may mediate the actions of certain oncogenes, such as ras, which cause intracellular increases in DAG and concomitant increases in protein kinase C. In addition, activation of protein kinase C leads to the expression of c-myc, c-fos, c-cis, c-fms, nuclear protooncogenes important in cell transformation. Overexpression of protein kinase C in NIH 3T3 cells causes altered growth regulation and enhanced tumorigenicity and in rat fibroblasts leads to anchorage-independent growth in soft agar. In these experiments, overexpression of protein kinase C in these cells resulted in tumor formation in animals receiving transplanted cells.

Several studies have shown increased expression of protein kinase C in certain tumor types such as breast and lung carcinomas. Activated protein kinase C has also been detected in human colon carcinomas although increased expression on the gene level was not seen. Topoisomerases are directly modulated by protein kinase C as substrates for the enzyme and protein kinase C inhibitors have been shown to potentiate the action of chemotherapy drugs such as cis-platinum.

New and more potent compounds which have been identified specifically as inhibitors of protein kinase C are showing early promise as therapeutic agents in inhibiting tumor growth in animal models.

Animal studies have shown that perhaps 50% or more of ischemic-related myocardial damages can be attributed to polymorphonuclear leukocytes (neutrophils) which accumulate at the site of occlusion. Damage from the accumulated neutrophils may be due to the release of proteolytic enzymes from the activated neutrophils or the release of reactive oxygen intermediates (ROI). Much of the "no reflow" phenomenon associated with myocardial ischemia is attributed to myocardial capillary plugging. The plugging of capillaries has been attributed to both aggregated platelets and aggregated neutrophils. Although

both cell types are aggregated during the ischemic event, the relative contribution of each to capillary plugging has not yet been established. It is well accepted that the damage by neutrophils to myocardial tissue proceeds through a cascade of events, one of the earliest being the bonding of activated neutrophils to damaged vascular endothelium. However, the binding of the neutrophils is significantly enhanced by their activation and this an even earlier event is the generation of molecules (such as cytokines, and chemotactic factors) which can function as activation stimuli. These molecules probably originate from damaged and aggregated platelets, from damaged vascular endothelium, or from the oxidation of plasma proteins or lipids by endothelial-derived oxidants.

Strategies for overcoming the deleterious effects of reactive oxygen intermediates have centered in the development of scavengers for the molecules. Superoxide dismutase (SOD) has been shown to be a particularly effective scavenger of superoxide, but suffers from a very short half-life in the blood. Several companies have tackled this problem by creating versions of this enzyme with increased half-lives by techniques such as liposome encapsulation or polyethylene glycol conjugation. Reports on the effectiveness of these new version are mixed. Catalase, a scavenger of hydrogen peroxide, and hydroxyl radical scavengers have also been tested and found to be effective to varying degrees. However, none of the strategies designed to scavenge reactive oxygen intermediates will prevent the aggregation of platelets, the release of chemotactic molecules, the activation and adherence of neutrophils to vascular endothelium, or the release of proteolytic enzymes from activated neutrophils.

The advantage of protein kinase C inhibitors as therapeutics for reperfusion injury is that they have been demonstrated to 1) block platelet aggregation and release of neutrophil activating agents such as PAF, 2) block neutrophil activation, chemotactic migration, and adherence

to activated or damaged endothelium, and 3) block neutrophil release of proteolytic enzymes and reactive oxygen intermediates. Thus these agents have the capability of blocking all three of the most significant mechanisms of pathogenesis associated with reperfusion injury and should thus have a decided therapeutic advantage.

The compounds of the invention may be made according to the methods in Scheme I, II and III, as shown in Figures 1, 2 and 3, respectively. Other synthetic routes known in the art may also be used. As used herein, the anthraquinone ring structure has the conventional ring numbering as illustrated in the Merck Index, Tenth Edition, Merck & Co., Inc. Rahway, New Jersey, 1983, pp.100-101.

When A is halo or comprises an oxirane ring with the adjacent oxygen atom, the compounds of the invention may be prepared according to the method in Scheme I shown in Figure 1. In Scheme I, reaction of diaminoanthraquinone 1 with epihalohydrin produces bis-(3-halo-2-hydroxypropylamino)-anthraquinone 2 which upon treatment with amine, such as diethylamine, at temperatures from room temperature to the boiling temperature of the chosen solvent, yields the product Ia. The epoxide analog Ib was obtained by treating Ia with base such as sodium hydroxide. An alternative procedure, shown in Scheme II in Figure 2, is the conversion of the bis-halohydrin intermediate 2 via basic treatment to bis-epoxide 3 followed by reaction of 3 with amine to yield Ib. Examples of compounds which may be prepared according to Schemes I and II are listed in Tables 1 and 2.

When A is a functional group other than halo or comprising an oxirane ring with the adjacent oxygen, the compounds of the invention listed in Table 1 may be prepared according to Scheme III as shown in Figure 3. For example, as shown in Figure 3, compound Ia from Scheme I is converted from the halo compound to the hydroxy compound Ic. The hydroxy compound is then used for the synthesis of

compounds having the formula of I wherein A is alkoxy, OCO (NR<sub>3</sub> R<sub>4</sub>) or SC (NH<sub>2</sub> = NR<sub>5</sub> using the method disclosed in Kocovsky *et al.* (1986) Tetrahedron Letters 27: 5521, the disclosures of which are hereby incorporated by reference as if fully set forth herein. Other methods known in the art are also suitable for the synthesis of compounds of the invention.

The optically active enantiomers of the compounds of the invention can be prepared according to Schemes 1 and 2, using optically active starting materials such as the commercially available glycidol or epichlorohydrin.

Pharmaceutically acceptable salts of the compounds of the invention are also useful in the methods of the invention. Pharmaceutically acceptable salts useful in the invention include salts of hydrochloric acid, hydrobromic acid, fumaric acid, oxalic acid, malic acid, succinic acid, pamoic acid, sulfuric acid and phosphoric acid.

Table 1

20	<u>Compound</u>	<u>R<sup>1</sup></u>	<u>R<sup>2</sup></u>	<u>X</u>	<u>A</u>	<u>m</u>	<u>n</u>	<u>MP (°C)</u>
	1a-1	Et	Et	H	Cl	1	1	106-
	1a-2	H	Pr	H	Cl	1	1	108(HCl)
	1a-3	H	CH <sub>2</sub> Ph	H	Cl	1	1	Hyg
25	1a-4	Octyl	Octyl	5-Cl	Br			
	1a-5	Et	Ph	6-Ph-O	I	2	3	
	1c-1	Et	Et	H	OH	1	1	Hyg
	1d-1	Et	Et	H	OCH <sub>3</sub>	1	1	
	1d-2	Me	Et	7-HO	O-(n-Bu)	2	2	
30	1e-1	Et	Et	H	O <sub>2</sub> CNH <sub>2</sub>	1	1	
	1e-2	Me	Me	8-Br	O <sub>2</sub> CNPr <sub>2</sub>	1	2	
	1f-1	Et	Et	H	SC(NH <sub>2</sub> )=NH <sub>2</sub>	1	1	
	1f-2	n-Bu	n-Bu	F	SC(NH <sub>2</sub> )=NMe <sub>2</sub>	3	3	
	1f-3	n-Bu	Me	5-COOH	C(NH <sub>2</sub> )=NPr <sub>2</sub>	1	2	



(Abbreviations used in Tables 1 and 2: Me - methyl; Et - ethyl; Pr - propyl; Ph - phenyl; Bu - butyl; Hyg - hygroscopic)

Table 2

5	<u>Compound</u>	<u>R<sup>1</sup></u>	<u>R<sup>2</sup></u>	<u>X</u>	<u>m</u>	<u>n</u>	<u>MP(°C)</u>
	1b-1	Et	Et	H	1	1	98(d)
	1b-2	H	Pr	H	1	1	
	1b-3	n-Bu	n-Bu	5-HO	2	1	
	1b-4	Pr	Me	5-Br	2	2	
10	1b-5	H	H	7-COOH	3	2	
	1b-6	Me	Me	8-Me	1	1	
	1b-7	n-Bu	Me	6-NMe2	1	1	

The compounds of the invention may be administered by any method that produces contact of the active ingredient with the agent's site of action in the body of a mammal including but not limited to oral, intravenous, and intraparenteral. The compounds of the invention may be administered singly, or in combination with other compounds of the invention, other pharmaceutical compounds, such as chemotherapy compounds, or other therapies, such as radiation treatment. The compounds are preferably administered with a pharmaceutically acceptable carrier selected on the basis of the selected route of administration and standard pharmaceutical practice.

The compounds of the invention are administered to mammals, preferably humans, in therapeutically effective amounts or concentrations which are effective to inhibit protein kinase C, or to inhibit tumor cell growth, inhibit inflammation of tissue, inhibit keratinocyte proliferation, inhibit oxidative burst from neutrophils or inhibit platelet aggregation. The dosage administered in any particular instance will depend upon factors such as the pharmacodynamic characteristics of the particular compound of the invention, and its mode and route of administration; age, health, and weight of the recipient; nature and extent

of symptoms; kind of concurrent treatment, frequency of treatment, and the effect desired. It is contemplated that the daily dosage of the compounds will be in the range of from about 5 to about 400 mg per kg of body weight, preferably from about 10 to about 200 mg per kg body weight, and more preferably from about 10 to about 50 mg per kg per day, and preferably administered in divided doses 2 to 4 times a day or in sustained release form.

The compounds of the invention may be administered as medicaments orally in solid dosage forms, such as capsules, tablets, and powders, or in liquid dosage forms, such as elixirs, syrups, and suspensions. They may also be administered parenterally in sterile liquid dosage forms.

The compounds of the invention may be formulated for use as medicaments into dosage forms according to standard practices in the field of pharmaceutical preparations. See *Remington's Pharmaceutical Sciences*, A. Osol, Mack Publishing Company, Easton, Pennsylvania, a standard reference text in this field.

For example, the compounds of the invention may be mixed with powdered carriers, such as lactose, sucrose, mannitol, starch, cellulose derivatives, magnesium stearate, and stearic acid for insertion into gelatin capsules, or for forming into tablets. Both tablets and capsules may be manufactured as sustained release products for continuous release of medication over a period of hours. Compressed tablets can be sugar coated or film coated to mask any unpleasant taste and protect the tablet from the atmosphere or enteric coated for selective disintegration in the gastrointestinal tract.

Liquid dosage forms for oral administration may contain coloring and flavoring to increase patient acceptance, in addition to a pharmaceutically acceptable diluent such as water, buffer or saline solution.

For parenteral administration, the compounds of the invention may be mixed with a suitable carrier or

diluent such as water, a oil, saline solution, aqueous dextrose (glucose), and related sugar solutions, and glycols such as propylene glycol or polyethylene glycols. Solutions for parenteral administration contain preferably  
5 a water soluble salt of the compound of the invention. Stabilizing agents, antioxidizing agents and preservatives may also be added. Suitable antioxidizing agents include sodium bisulfite, sodium sulfite, and ascorbic acid, citric acid and its salts, and sodium EDTA. Suitable  
10 preservatives include benzalkonium chloride, methyl- or propyl-paraben, and chlorbutanol.

### Examples

The following are specific examples which are illustrative of the present invention and are not intended  
15 to limit the scope of the invention.

#### Example 1 1,4-Bis-(3-Chloro-2-Hydroxypropylamino)-9, 10-Anthracenedione

Epichlorohydrin (84.5 mL, 1.08 mol) was added to a solution of 1,4 - diaminoanthraquinone (Aldrich Chemical  
20 Company, Milwaukee, Wisconsin) (10g, 42 mmol) in glacial acetic acid (200 mL) at room temperature. The solution was stirred for 30 minutes at 90°C and the solvent was removed under reduced pressure. The residue was purified by flash column chromatography (silica gel, CH<sub>2</sub>Cl<sub>2</sub>:MeOH/25:1) and  
25 recrystallized from CH<sub>2</sub>Cl<sub>2</sub>-Ether-Hexanes to give a pure blue solid of the title compound (12.46 g, 70%), mp 167-169° C:  
<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 3.21 (broad, 2H, OH), 3.5-3.6 (m, 6H, CHCH<sub>2</sub>), 3.75 (d, J=3Hz, 4H, CH<sub>2</sub>), 4.19 (broad, 2H, NH), 7.03 (d, J=3.2 Hz, 2H, ArH (2, 3)), 7.69 (m, 2H, ArH (6, 7)),  
30 8.27 (m, 2H, ArH (5, 8)). Anal. Calcd for C<sub>20</sub>H<sub>20</sub>O<sub>4</sub>N<sub>2</sub>Cl<sub>2</sub> x .25 H<sub>2</sub>O: C, 56.15; H, 4.83; N, 6.55. Found: C, 56.18; H, 4.76; N, 6.29.

Example 2 1-(3-Diethylamino-2-Hydroxypropylamino)-4-(-  
Chloro-2-Hydroxypropylamino)-9, 10 Anthracenedione  
(Compound 1a-1 in Table 1)

Diethylamine (3.15 mL, 60.86 mmol) was added  
5 dropwise over 1.5 hours to a solution of 1,4-bis-(3-Chloro-  
2-hydroxypropylamino)-9, 10 anthracenedione (12.88 g, 30.43  
mmol) the compound of Example 1, compound 2 in Schemes I  
and II. in ethanol (200 mL) under nitrogen atmosphere.  
The solution was stirred for 48 hours at reflux and the  
10 solvent was removed under reduced pressure. The residue  
was purified by flash column chromatography (silica gel,  
CH<sub>2</sub>Cl<sub>2</sub>:MeOH / 25:1) and recrystallized from CH<sub>2</sub>Cl<sub>2</sub>-ether-  
hexanes to give a blue solid of the title compound 1-(3-  
diethylamino-2-hydroxypropylamino)-4-(-chloro-2-  
15 hydroxypropylamino)-9, 10 anthracenedione which is compound  
1a-1 in Table I (8.1 g, 60%), mp 165° C: <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ  
1.10 (t, J=7Hz, 6H, CH<sub>3</sub>), 2.63 (m, 4H, CH<sub>2</sub>), 2.74 (m, 2H,  
CH<sub>2</sub>), 3.44 (m, 2H, CH<sub>2</sub>), 3.56 (m, 1H, CH), 3.63 (m, 1H, CH),  
3.73 (m, 2H, CH<sub>2</sub>), 4.17 (broad, 1H, NH), 4.19 (broad, 1H,  
20 NH), 7.22 (s, 2H, ArH (2, 3)), 7.68 (m, 2H, ArH (6, 7)),  
8.3 (m, 2H, ArH (5,8)). Anal. Calcd for C<sub>24</sub>H<sub>30</sub>O<sub>4</sub>N<sub>3</sub>Cl \*1.25  
H<sub>2</sub>O: C, 59.74; H, 6.69; N, 8.71. Found: C, 59.50; H, 6.27;  
N, 8.50.

Example 3 1-(3-Diethylamino-2-Hydroxypropylamino)-4-(3-  
25 Chloro-2-Hydroxypropylamino)-9, 10-Anthracenedione  
Hydrochloride Salt (Compound 1a-1 in Table 1)

Concentrated HCl (8 drops) was slowly added to a  
solution of 1-(3-diethylamino-2-hydroxypropylamino)-4-(3-  
chloro-2-hydroxypropylamino)-9, 10-anthracenedione (0.531  
30 g, 2.36 mmol) in acetone-dichloromethane (50%, 15 mL). A  
color change from purple to red was observed. The solid  
was obtained by filtration, mp 106-108° C, extremely  
hygroscopic. <sup>1</sup>H NMR (D<sub>2</sub>O) δ 1.40 (t, J=7.3 Hz, 6H, CH<sub>3</sub>),  
2.90 (m, 4H, CH<sub>2</sub>), 3.29 (m, 4H, CH<sub>2</sub>), 3.38 (m, 3H, CH<sub>2</sub>, CH),  
35 3.73 (m, 3H, CH<sub>2</sub>, CH), 3.92 (broad, 1H, NH), 4.15 (broad,  
1H, NH), 6.23 (s, 2H, ArH (2, 3)), 7.26 (m, 2H, ArH (6,  
7)), 7.32 (m, 2H, ArH (5, 8)). Anal. Calcd for

$C_{24}H_{30}ClN_3O_4 \cdot 2HCl \cdot 1.5 H_2O$ : C, 51.48; H, 6.30; N, 7.50.

Found: C, 51.70; H, 6.35; N, 7.41.

Example 4 1,4-Bis-(2, 3-Epoxypropylamino)-9, 10-Anthracenedione

5 Sodium hydroxide (3.5 g) was added to a solution of 1,4-bis-(3-chloro-2-hydroxypropylamino)-9, 10-anthracenedione (8.3 g, 19.8 mmoles), the compound of Example 1, in MeOH (500 mL) at 60° C. The resulting blue solution was stirred at room temperature for 4 hours, and  
10 the solvent was removed under reduced pressure. The residual solid was then purified by flash column chromatography (silica gel,  $CH_2Cl_2$ : MeOH / 25:1). Recrystallization from  $CH_2Cl_2$ : hexanes yielded the title compound 1,4-bis-(2, 3-epoxypropylamino)-9, 10-anthracenedione which is compound 3 in Scheme 2 (6.2g,  
15 89.37%), mp 189-190° C:  $^1H$  NMR ( $CDCl_3$ )  $\delta$  2.74 (m, 1H,  $CH_2$ ), 2.87 (m, 1H,  $CH_2$ ), 3.26 (m, 1H, CH), 3.57 (m, 1H  $CH_2$ ), 3.78 (m, 1H,  $CH_2$ ), 7.32 (s, 2H, Ar (2, 3)), 7.71 (dd, J=3.2 Hz, 5.5 Hz, 2H, Ar (6, 7)), 8.34 (dd, J=3.2 Hz, 5.5 Hz, 2H, Ar  
20 (5, 8)), 10.76 (s, 2H, NH). Anal. Calcd for  $C_{20}H_{18}O_4N_2 \cdot .25 H_2O$ : C, 67.66; H, 5.25; N, 7.93. Found: C, 67.72; H, 5.55; N, 7.54. IR: 3400, 3070, 2910, 1640, 1220, 900.

Example 5 1-(3-Diethylamino-2-Hydroxypropylamino)-4-(2, 3-Epoxypropylamino)-9, 10-Anthracenedione (Compound 1b-1 of Table 1)

25 Potassium hydroxide (0.3 g, 5.36 mmole) was added to a solution of 1-(3-diethylamino-2-hydroxypropylamino)-4-(3-chloro-2-hydroxypropylamino)-9, 10-anthracenedione hydrochloride salt (1 g, 1.78 mmole), the compound of Example 3, in methanol. The mixture was stirred at room temperature for 48 hours. Solvent was removed under reduced pressure and the residue was purified by flash column chromatography (silica gel, chloroform with a methanol gradient up to a ratio of 10:1) to give the title  
30 product 1-(3-diethylamino-2-hydroxypropylamino)-4-(2, 3-epoxypropylamino)-9, 10-anthracenedione which is compound 1b-1 of Table 1 (391 mg, 52%), mp 98° C: NMR ( $CDCl_3$ )  $\delta$  1.06

(t, J=7.1 Hz, 6H, CH<sub>3</sub>), 2.62 (m, 7H, CH<sub>2</sub>), 2.87 (m, 1H, CH<sub>2</sub>), 3.27 (m, 1H, CH<sub>2</sub>), 3.49 (m, 2H, CH<sub>2</sub>), 3.59 (m, 1H, CH<sub>2</sub>), 3.77 (m, 1H, CH), 3.96 (m, 1H, CH), 7.35 (s, 2H, Ar (2, 3)), 7.71 (m, 2H, Ar (6, 7)), 8.35 (m, 2H, Ar (5, 8)), 10.80 (b, 1H, NH), 10.92 (b, 1H, NH); Anal. Calcd for C<sub>24</sub>H<sub>29</sub>N<sub>3</sub>O<sub>4</sub>: C, 68.1; H, 6.90; N, 9.92. Found: C, 67.86; H, 6.96; N, 9.85.

**Example 6 1-(3-Diethyl-2-Hydroxypropylamino)-4-(2, 3-Dihydroxypropylamino)-9, 10-Anthracenedione Hydrochloride Salt (HCl salt of compound 1C-1 of Table 1)**

Para-toluenesulfonic acid (272 mg, 1.43 mmole) was added to a solution of 1-(3-diethylamino-2-hydroxypropylamino)-4-(2, 3-epoxypropylamino)-9, 10 anthracenedione (120 mg, 0.28 mmole), the compound of Example 5, in methanol-water (1:1, 20 mL). The mixture was stirred for 16 hours at room temperature. The solvent was removed under reduced pressure and the residue was treated with chloroform-water. The organic layer was dried over magnesium sulfate and, after the removal of solvent, the residue was purified by column chromatography (silica gel, CH<sub>2</sub>Cl<sub>2</sub> with an increasing methanol gradient to give the product as a free base. Hydrochloride gas was passed through a methanolic solution of the free base until a color change was noted. The title compound 1-(3-diethyl-2-hydroxypropylamino)-4-(2, 3-dihydroxypropylamino)-9, 10 anthracenedione hydrochloride salt was then isolated by filtration and was found to be extremely hygroscopic. NMR (CDCl<sub>3</sub>) δ 1.06 (t, J=7.1 Hz, 6H, CH<sub>3</sub>), 2.72-2.55 (m, 6H, CH<sub>2</sub>), 3.19 (m, 4H, CH<sub>2</sub>), 3.31 (b, 3H, OH), 3.81 (m, 2H, CH), 3.87 (b, 1H, OH), 3.92 (m, 2H, CH<sub>2</sub>), 4.11 (b, 1H, OH), 6.65 (m, 2H, Ar (2, 3)), 7.52 (m, 2H, (6, 7)), 8.08 (m, 2H, (5, 8)), 10.73 (m, 2H, NH);

**Example 7 Protein Kinase C Inhibition**

The protein kinase C (PKC) assay is designed to duplicated the in vivo conditions required for protein kinase C function. Therefore, pH, salt and cofactor

concentrations are similar to physiologic levels. Histone H1 (lysine rich) is used in the assay as the phosphorylation acceptor protein because it is readily available and serves as a good substrate for protein kinase C. The enzyme is prepared from rat brain and is purified to apparent homogeneity as determined by a single band on silver stained SDS-polyacrylamide. Studies on the mechanism of regulation of protein kinase C by phospholipids, DAG and  $\text{Ca}^{+2}$  have been hampered by the physical properties of the lipid cofactors. In the screening assay, phosphatidylserine (PS) and DAG are co-sonicated to form unilamellar and multilamellar vesicles. The concentration of lipids in the assay are suboptimal to maximize the detection potential of the assay for inhibitors. Potential inhibitor compounds are added to the assay in dimethylsulfoxide at three concentrations to give final inhibitor concentrations of 4.3, 43 and 218  $\mu\text{M}$ , respectively. The assay is started with the addition of enzyme and stopped after 10 min by the addition of 25% trichloroacetic acid (TCA) and 1.0 mg/ml bovine serum albumin (BSA). The radioactive histone product is retained and washed on glass fiber filters that allow the unreacted  $^{32}\text{P}$ -ATP to pass through. The amount of phosphorylation is determined by the radioactivity measured in a scintillation counter. Controls are included in every assay to measure background activity in the absence of enzyme, activity in the absence of lipids and the maximum enzyme activity with saturating levels of the activator lipids. Assay components and concentrations are given in Table 3.

Table 3

<u>Assay Component</u>	<u>Concentration</u>
Hepes pH 7.5	20 mM
$\text{MgCl}_2$	20 mM
$\text{CaCl}_2$	100 $\mu\text{M}$
EGTA	95 $\mu\text{M}$
Histone H1	200 $\mu\text{g/ml}$
Phosphatidylserine	40 $\mu\text{g/ml}$
Diacylglycerol	1.8 $\mu\text{g/ml}$

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Protein Kinase C  
 $\gamma$ -<sup>32</sup>P-ATP

0.6  $\mu$ g/ml  
20  $\mu$ M

Results of the protein kinase C assay are shown in Table 4 in the column labeled protein kinase C. Results are shown as IC<sub>50</sub>, which is the concentration of test compound needed to inhibit 50% of the protein kinase C activity as compared with levels of protein kinase C activity in controls. As shown in Table 4, all but one of the compounds tested was able to inhibit fifty per cent of protein kinase C activity at concentrations less than 200  $\mu$ M. Compound 1b-1 (base) had the lowest IC<sub>50</sub> of 110  $\mu$ M, whereas compound 1a-1 (HCl salt) had the highest IC<sub>50</sub> of the test compounds at 230  $\mu$ M.

Table 4

IC<sub>50</sub> ( $\mu$ M)

<u>Exp.</u>	<u>PKC</u>	<u>PKA</u>
1a-1(base)	180	n.e.
1a-1(HCl)	230	n.e.
1a-2(HCl)	160	n.e.
1a-3(HCl)	110	n.e.
1c-1(HCl)	180	n.e.
1b-1(base)	110	n.e.

(In Table 4, n.e. = no effect)

Example 8    cAMP Dependent Protein Kinase (PKA) Assay

Compounds found to be inhibitors of protein kinase C were also tested for inhibitory activity against cAMP dependent protein kinase (PKA). This enzyme, like protein kinase C, plays an important role in cell-cell communication and is activated by a second messenger, cAMP. Secondary screening against PKA is useful for ascertaining the selectivity of the compounds of the invention. The



standard assay conditions are given in Table 5. The catalytic subunit of PKA (Sigma Chemical Company, St. Louis, Missouri) is mixed with buffer before addition of the inhibitor in dimethylsulfoxide (DMSO). The assay is started by the addition of  $^{32}\text{P}$ -ATP and the reaction is allowed to proceed for 10 min before stopping with 25% trichloroacetic acid (TCA) and 1.0 mg/ml bovine serum albumin (BSA). The phosphorylated protein is isolated by filtration and the radioactivity is counted in a beta scintillation counter.

Table 5

	<u>Assay Components</u>	<u>Concentration</u>
	Hepes pH7.5	20 mM
	Histone	200 $\mu\text{g/ml}$
15	Dithiothreitol	32 $\mu\text{g/ml}$
	Protein Kinase	2.6 $\mu\text{g/ml}$
	$\gamma$ - $^{32}\text{P}$ -ATP	20 $\mu\text{M}$

Results of the PKA assay are shown in Table 4 (in Example 7) in the column labeled PKA. As shown in Table 4, the compounds of the invention that were tested had no effect on PKA. The tested compounds of the invention are selective for protein kinase c, and have no effect on cAMP dependent protein kinase. The compounds of the invention should thus have no effect on the metabolic pathways associated with stimulation of protein kinase by cAMP.

Example 9 Human Tumor Growth Inhibition

MCF-7 a human breast tumor cell line, and MCF-7/ADR an adriamycin resistant line of MCF-7 cells were obtained from the National Cancer Institute, Frederick, Maryland. NHEK a human keratinocyte cell line was obtained from Clonetic Corp., San Diego, California. 8226 a human myeloma cell line, 8226/MT a Mitoxantrone resistant line of 8226 cells, and 8226 ADR an adriamycin resistant line of 8226 cells were obtained from Dr. Bill Dalton, Arizona Cancer Center, Tuscon, Arizona.

Human tumor cells are trypsinized (0.05% trypsin, GIBCO), counted with a hemacytometer and seeded at a concentration of 10,000 cells/well in a 96 well microtiter plate. After allowing cells to attach to the surface overnight, the culture medium is aspirated and replaced with 100  $\mu$ l of fresh medium. Test agents are diluted to determine dose response at 2X final concentration and added in quadruplicate at 100  $\mu$ l/well to bring the total volume of each well to 200  $\mu$ l. The microtiter plate is then incubated at 37°C 5% CO<sub>2</sub> overnight (18-24 hrs) before <sup>3</sup>H-thymidine is added at a concentration of 0.5  $\mu$ Ci/well in 50  $\mu$ l culture medium. The plate is incubated again for 4 hrs under the same conditions as above. Supernatant is then aspirated and 50 $\mu$ l trypsin (0.05%, GIBCO) is added to each well. Cells are checked microscopically to determine detachment from surfaces, and plates are then harvested with a cell harvester (PHD, Cambridge Technology, Inc.) Filter papers corresponding to wells are placed in scintillation vials and counted to determine the amount of <sup>3</sup>H-thymidine incorporated by the cells. Test agent response is compared to a positive control of cell wells with culture media only to determine the IC<sub>50</sub>. IC<sub>50</sub> is the concentration of test compound required to inhibit fifty per cent of the incorporation of <sup>3</sup>H-thymidine into proliferating cells not exposed to test agent. Uptake of <sup>3</sup>H-thymidine is a standard test for measuring the metabolism of cells. Cells which are actively proliferating take up <sup>3</sup>H-thymidine, whereas cells that are not proliferating take up <sup>3</sup>H-thymidine at much slower rates or not at all. Test agents that inhibit the uptake of <sup>3</sup>H-thymidine thus slow the growth of cells.

Results of these experiments are shown in Tables 6 and 7. As shown in Table 6, both compounds 1a-1 and 1b-1 were able to inhibit fifty per cent of <sup>3</sup>H-thymidine uptake at concentrations less than 1  $\mu$ M (IC<sub>50</sub>=0.25  $\mu$ M and 0.025  $\mu$ M, respectively). The IC<sub>50</sub> of compound 1b-1 especially compares well with the IC<sub>50</sub> of adriamycin.

When compounds of the invention were tested with adriamycin resistant cell line MCF-7/ADR, the  $IC_{50}$  of the test compounds increased to amounts from 0.06 to 16  $\mu M$ , resulting in a resistance ratio of  $IC_{50}$  for MCF-7/ADR cells to  $IC_{50}$  for MCF-7 cells of from 1.9 to 2.4. In contrast, the  $IC_{50}$  for MCF-7/ADR cells cultured with adriamycin was 4  $\mu M$ , with a ratio of 200. The  $IC_{50}$  for MCF-7/ADR cells cultured with Mitoxantrone was also 4  $\mu M$ , but the ratio of  $IC_{50}$  for MCF-7/ADR cells to  $IC_{50}$  for MCF-7 cells was 800.

Similar results were obtained when the compounds were tested with cell lines 8226, 8226/MT (Mitoxantrone resistant) and 8226/ADR (adriamycin resistant). Compounds 1a-1 and 1b-1 were able to inhibit  $^3H$ -thymidine uptake at very low concentrations. The  $IC_{50}$  for compound 1a-1 was 1  $\mu M$ . The  $IC_{50}$  for compound 1b-1 was 0.06  $\mu M$ . The  $IC_{50}$  for the other compounds tested were 10.2 and 15  $\mu M$ . When compounds 1a-1 and 1b-1 were tested with cell line 8226/MT the  $IC_{50}$  was 0.24 and 0.008, respectively, with a ratio of  $IC_{50}$  for 8226 cells to  $IC_{50}$  for 8226/MT cells of 0.24 and 0.13 respectively. These results compared favorably with the results obtained from cells cultured with adriamycin or Mitoxantrone. The ratio of  $IC_{50}$  for 8226/MT cells to  $IC_{50}$  for 8226 cells for cells cultured with adriamycin was 1  $\mu M$ . The ratio of  $IC_{50}$  for 8226/MT cells to  $IC_{50}$  for 8226 cells for cells cultured with Mitoxantrone was 3.5.

Compounds tested with adriamycin resistant 8226 cells also showed a similar results. The test compounds were not cross-resistant with adriamycin as shown by the ratio of  $IC_{50}$  for 8226/ADR cells to  $IC_{50}$  for 8226 cells ranging from 0.08  $\mu M$  to 1.4  $\mu M$  for test compounds compared with resistance ratios of 11.4  $\mu M$  and 6  $\mu M$  for adriamycin and mitoxantrone, respectively.

These results show that the compounds of the invention are effective in inhibiting the proliferation of tumor cells and are not cross-resistant with adriamycin or mitoxantrone.

Table 6

<u>Compound</u>	<u>MCF-7</u>	<u>MCF-7/ADR</u>	<u>Resistance Ratio</u>
1a-1	0.25	0.5	2
5 1a-2	7.4	16	2.2
1a-3	7.4	14.1	1.9
1b-1	0.025	0.06	2.4
ADR	0.02	4	200
MT	0.005	4	800

10

TABLE 7

<u>Compound</u>	<u>8226</u>	<u>8226/MT</u>	<u>Resistance Ratio</u>	<u>8226/ADR</u>	<u>Resistance Ratio</u>
1a-1	1	0.24	0.24	0.08	0.08
1a-2	10.2			14.1	1.38
15 1a-3	15			16.3	1.08
1b-1	0.06	0.008	0.13	0.016	0.26
1c-1	18			25	1.4
ADR	0.05	0.05	1	0.57	11.4
MT	0.03	0.105	3.5	0.18	6

20 Example 10 Human Keratinocyte Inhibition

Proliferating keratinocytes (NHEK cells purchased from Clonetics, Inc. San Diego, California) in second passage were grown in Keratinocyte Growth Medium (KGM) (Clonetics, Inc.) Cells were trypsinized with 0.025%

trypsin (Clonetics), counted with a hemacytometer (Scientific Products), and seeded at a concentration of 2,500 cells/well in a 96 well microtiter plate. After allowing cells to attach to the surface overnight, the culture medium is aspirated and replaced with 100  $\mu$ l of fresh KGM. Test agents were evaluated and  $IC_{50}$ 's are determined according to the  $^3H$ -thymidine incorporation procedures described as in Example 9.

The results of these experiments are shown in Table 8. These results indicate that compounds of the invention are active against human keratinocytes, and will be useful in treating topical inflammatory conditions such as psoriasis and other conditions where hyperproliferation of keratinocytes is a symptom.

Table 8

Compound	$IC_{50}$ ( $\mu$ M)
1a-1	0.16
1b-1	0.04

#### Example 11 Neutrophil Superoxide Anion ( $O_2^-$ ) Release

##### Assay

Neutrophils were isolated from whole blood collected from human volunteers. All reagent materials were obtained from Sigma Chemical Company with the exception of isotonic saline (Travenol Lab., Inc. Deerfield, Illinois) and lymphocyte separation medium (Organon Teknika, Durham, North Carolina).

##### Neutrophil Isolation

Whole blood was drawn and mixed with sodium heparin (final conc. 10 units/ml) to prevent clotting. An equal volume of dextran (3.0%) in isotonic saline was added, mixed, and allowed to settle for 30 min to bind red blood cells (RBC). The supernatant was removed, underlayered with lymphocyte separation medium and centrifuged for 40 min at 400 xg in a centrifuge (Beckman GPR, Norcross, Georgia). The pellet was alternately

resuspended in 0.2% and 1.6% NaCl to lyse RBCs before washing with Hank's Balanced Salt Solution (HBSS). The washed pellet was resuspended in 10 ml HBSS and placed on ice before counting on a hemacytometer.

5

#### Assay Procedure

The neutrophil cell concentration is adjusted to  $2 \times 10^6$  cells/ml with HBSS before adding 0.8 ml cells to 12 X 75 mm polypropylene test tubes (Fisher Scientific). Test agents are diluted to determine dose response and added at 10 10X final concentration at a volume of 0.1 ml/tube in duplicate. Then 10X concentrations of cytochrome C (15 mg/ml) with catalase (3000 units/ml) either alone or containing 25 ng/ml phorbol 12-myristate 13-acetate (PMA) are added at a volume of 0.1 ml/tube and incubated at 37° C 15 for 30 min before stopping the reaction by placing tubes on ice. Tubes are then centrifuged at 900 xg for 10 min, 0.5 ml supernatant is removed and added to 0.5 ml H<sub>2</sub>O in a microcuvette. Optical density (OD) of cytochrome c is read in a spectrophotometer (Shimadzu) at 550 nm. The  $\Delta OD$  of 20 cytochrome c is obtained between PMA-stimulated and non-stimulated tubes, and the dose responses of the test agents are compared to the positive controls (which contain HBSS in place of test agents). PMA stimulates  $O_2^-$  production which reduces cytochrome c. Reducing cytochrome c 25 increases its absorbance, and the change in OD of cytochrome c is proportional to the amount of  $O_2^-$  produced by PMA stimulation. Inhibition of the  $O_2^-$  burst by test compounds of the invention is seen as a reduction in the change in optical density. Inhibition is expressed as  $IC_{50}$  30  $\mu M$  and is the amount of test compound that will inhibit fifty per cent of the PMA-stimulated respiratory outburst, i.e.  $O_2^-$  production.

The tested compounds were able to inhibit  $O_2^-$  production by PMA-stimulated neutrophils. As shown in 35 Table 9, compound 1a-1(base) had an  $IC_{50}$  greater than 6.25  $\mu M$ . Compounds 1a-1(HCl) and 1b-1(base) had  $IC_{50}$  of greater than 12.5  $\mu M$  and 25  $\mu M$ , respectively.

Table 9  
IC<sub>50</sub> (μM)

	<u>Exp.</u>	<u>Human Neutrophil</u>
5	1a-1(base)	>6.25
	1a-1(HCl)	>12.5
	1b-1(base)	>25

Example 12 - Tumor Assays

10            P388 Leukemia Tumors. Mice were young adult,  
pathogen-free, BALA/c x DBA/2 F1 (i.e., CD2F1) females  
purchased from Charles River Laboratories. The tumor line  
was the P388 murine leukemia, ascites form, obtained from  
the National Cancer Institute (NCI) Tumor Repository  
15 (Frederick, MD). The tumor was maintained in the serial  
passage in mice by periodic intraperitoneal (ip)  
implantation. For this experiment, 10<sup>6</sup> hemacytometer-  
counted P388 murine leukemia cells were implanted ip into  
mice on Day 0. The mice were randomized into one untreated  
20 control group of 20 and 15 test groups of six mice each.  
All drug treatment was delivered ip on the basis of the  
average body weight of each group. All drugs were  
dissolved in sterile saline for injection, and all drugs  
were soluble at all dosages prepared. Drugs, dosages, and  
25 treatment schedules used were: mitoxantrone, a positive  
control drug, injected once every four days beginning on  
Day 1 for a total of three injections at 1.2, 1.8, and 2.7  
mg/kg/dose; Compound 1a-1 injected once every four days  
(Q4Dx3) beginning on Day 1 for a total of three injections  
30 at 100, 150 and 225 mg/kg/dose; Compound 1a-1 injected once  
daily for nine days (Q1Dx9) beginning on Day 1 at 67, 100,  
and 150 mg/kg/dose; and Compound 1b-1 delivered on the same  
treatment schedules as Compound 1a-1, but at dosages of  
10.7, 16 and 24 mg/kg/dose and 3.5, 5.3, and 8 mg/kg/dose,  
35 respectively. Body weights were recorded on Days 1, 5, 9,  
11, and 18. Deaths were recorded daily. Dying mice were

necropsied, and the presence or absence of ascites or splenomegaly was recorded. Drug effectiveness was assessed on the basis of the survival time. Results were expressed as a percentage of the control survival time (Survival Time =  $T/C \times 100\%$ ; T = test animals; C = control animals). The criterion for effectiveness was determined by:  $T/C \times 100 \geq 125$  percent.

TABLE 10

% T/C (Dose, Schedule)

10	<u>Compound</u>	<u>p388 Leukemia</u>
	<u>1a-1</u>	130 (150mg/kg, Q1Dx9)
	<u>1a-1</u>	140 (67mg/kg, Q1Dx9)
	<u>1b-1</u>	140 (16mg/kg, Q4Dx3)

As shown in Table 10, compounds 1a-1 and 1b-1 effectively increased the survival time of mice treated with the compounds at all dosages and schedules.

Melanotic Melanoma B16 Test. The animals used were  $B_6C_3F_1$  mice, all of one sex, weighing a minimum of 18 g for males and 17 g for females and all within a 4 g weight range at the start of the test. The test group comprised 9 or 10 mice. The tumor was implanted in each of the test mice by the subcutaneous injection of 0.5 ml of a tumor homogenate prepared by homogenizing a 1 g portion of melanotic melanoma in 10 ml of cold physiological saline.

Compound 1a-1 suspended in hydroxypropylcellulose was administered intraperitoneally at a dose of 100 mg/kg once daily for nine consecutive days starting on Day 1 relative to the day of tumor inoculation (Day 0). The control mice received injections of hydroxypropylcellulose vehicle only.

The mice were weighed and survivors were recorded on a regular basis for sixty days. The median survival times and the ratio of the median survival times for treated (T) to control (C) mice were calculated. The median survival time of the nontreated tumored mice ranged from 15 to 17 days. Drug effectiveness was assessed on the basis of



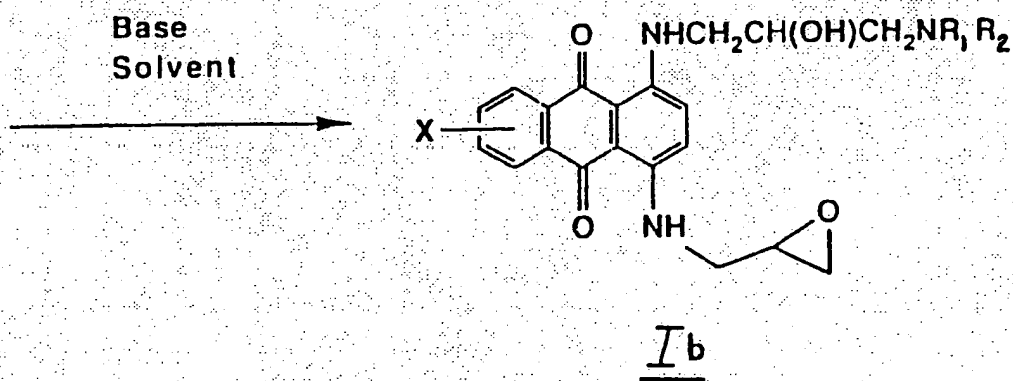
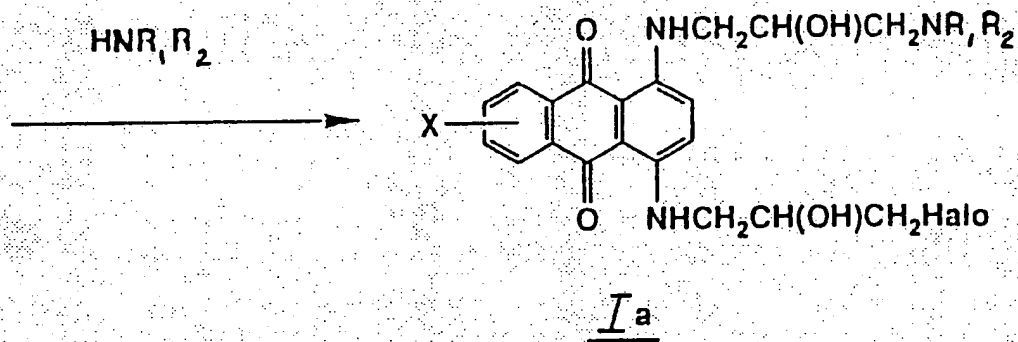
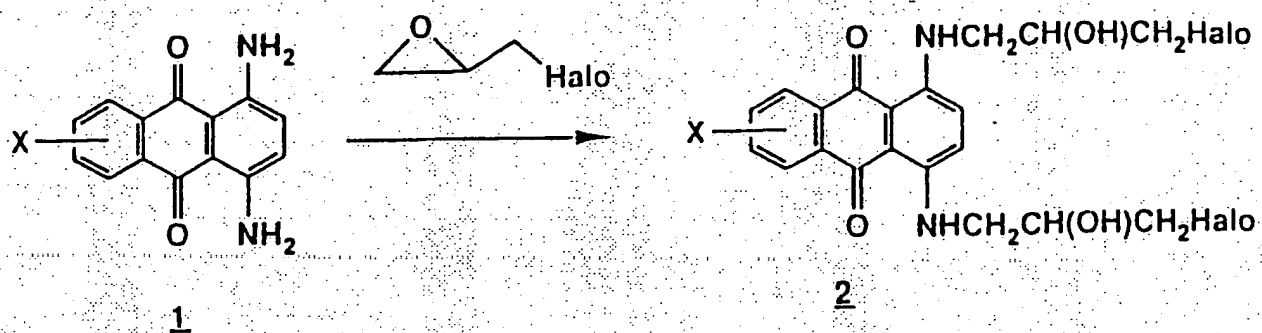


Figure 1

- 31 -

survival time. Results were expressed as a percentage of the control survival time (Survival Time T/Cx100%; T = test animals; C = control animals). The criterion for effectiveness was determined by  $T/Cx100 \geq 125$  percent.

- 5                   % T/C for tested compound 1a-1 was 129,  
indicating that compound 1a-1 is effective for increasing survival time of the mice treated with the compound.

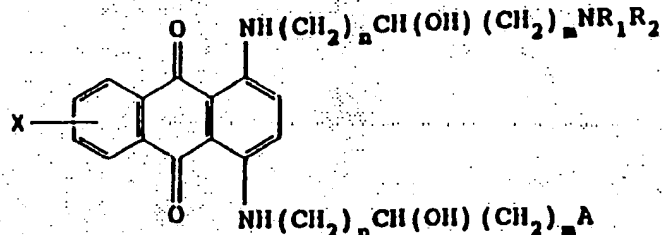
MCF-7 Human Mammary Carcinoma Xenografts Mice were young adult, pathogen-free, NCrOnu (athymic nude) females purchased from Taconic Farms. MCF-7 human mammary carcinoma cells were obtained from the NCI Tumor Repository, expanded in culture, adapted to growth as xenografts in nude mice, and maintained in serial passage in nude mice as subcutaneous (sc) tumors. All mice except one estrogen-free control group (10 mice) received a controlled-release estradiol pellet (Innovative Research of America, Toledo, OH) implanted subcutaneously on Day 0. Tumor fragments (approximately 30 mg each) were implanted subcutaneously by trocar on the flank opposite the estradiol implant on Day 0. The estrogen-free controls were also implanted with tumor fragments. Estrogen-implanted mice were randomized into a control group of 30 mice and five test groups of 10 mice each. Both drugs were dissolved in sterile saline for injection, and both drugs were soluble at the dosages prepared. The five test groups received drug treatment as follows: adriamycin, a positive control drug, was injected once every four days beginning on Day 1 for a total of three injections at 3.35 and 5 mg/kg/dose. Compound 1b-1 was injected once every four days beginning on Day 1 for a total of five injections at 16, 25 and 40 mg/kg/dose. All drug treatment was administered intravenously (iv) to mice via the tail vein. Estrogen-free and estrogen-implanted controls were untreated. Body weights were recorded on Days 1, 5, 9, 13, 17, 21, 23 and 30. Deaths were recorded daily. Mice were palpated daily for the appearance of sc tumors. Tumors

were measured twice weekly in two dimensions by calipers. Tumor volume was calculated using the formula for a prolate ellipsoid, and volume was converted to mass assuming unit density. Therapeutic response was assessed on the basis of tumor growth delay (T-C), tumor free survival, and overall survival. Drug toxicity was assessed on the basis of body weight loss and early (relative to the completion of treatment) deaths.

Figure 4 shows a graph of the median tumor weight (mg) vs. the days post implant of the tumors. Results for the control group of mice (estrogen IMP.) are shown by solid circles; the results for compound 1b-1 are shown by solid squares. As shown in Figure 4, tumor growth of 1b-1 treated mice was stabilized at about day 30 at a weight significantly lower than the weight of the control mice. The median tumor weight for the treated mice remained stable until about day 38 when tumor regrowth began to increase.

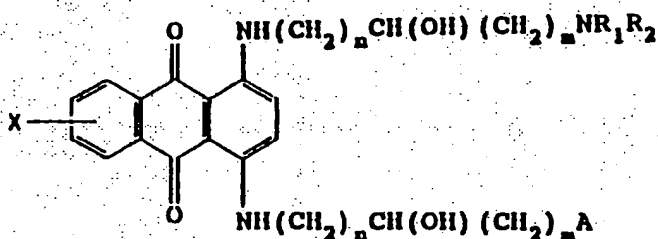
Claims

1. A substituted anthraquinone having the formula



- wherein  $R_1$  and  $R_2$  are independently H,  $C_1$ - $C_{10}$  alkyl, aryl, arylalkyl, alkylaryl, or  $R_1$  and  $R_2$  taken together with the adjacent nitrogen atom N form a substituted or unsubstituted cyclic ring which may optionally contain additional heteroatoms;  $n$  and  $m$  are independently 1, 2, or 3; A is Halogen, OH, alkoxy,  $\text{OCO}(\text{NR}_3\text{R}_4)$ ,  $\text{S}-\text{C}(\text{NH}_2)=\text{NR}_5$ , or when  $m = 1$ , comprises an oxirane ring with the adjacent oxygen atom;  $R_3$ ,  $R_4$ , and  $R_5$  are independently H, alkyl, or aryl; X is H, OH,  $\text{NR}_6\text{R}_7$ , Cl, Br, I, F, alkyl, aryl, alkoxy, aryloxy,  $\text{COOR}_8$ , or  $\text{CONR}_9\text{R}_{10}$ ; and  $R_6$ ,  $R_7$ ,  $R_8$ ,  $R_9$ , and  $R_{10}$  are independently H, lower alkyl, or aryl.
2. The substituted anthraquinone of claim 1 wherein  $R_1$  and  $R_2$  independently are H or lower alkyl;  $n$  and  $m$  are independently 1 or 2; A is halogen,  $\text{OCO}(\text{NR}_3\text{R}_4)$ ,  $\text{SC}(\text{NH}_2)=\text{NR}_5$ , or when  $m = 1$ , comprises an oxirane ring with the adjacent oxygen atom;  $R_3$ ,  $R_4$ , and  $R_5$  are independently H or alkyl; and X is H, HO, Cl, Br, I, F, or alkoxy.
3. The substituted anthraquinone of claim 2 wherein  $R_1$  and  $R_2$  independently are lower alkyl;  $m$  and  $n$  are 1; A is Cl, Br,  $\text{OCO}(\text{NR}_3\text{R}_4)$ ,  $\text{SC}(\text{NH}_2)=\text{NR}_5$ , or when  $m = 1$ , comprises an oxirane ring with the adjacent oxygen atom;  $R_3$ ,  $R_4$ , and  $R_5$  are H; and X is H or OH.
4. A pharmaceutical composition for inhibiting protein kinase C comprising a substituted anthraquinone of claim 1 and a pharmaceutically acceptable carrier or diluent.

5. A pharmaceutical composition for inhibiting protein kinase C comprising a substituted anthraquinone of claim 2 and a pharmaceutically acceptable carrier or diluent.
6. A pharmaceutical composition for inhibiting protein kinase C comprising a substituted anthraquinone of claim 3 and a pharmaceutically acceptable carrier or diluent.
7. A method of inhibiting protein kinase C which comprises contacting protein kinase C with an inhibitory amount of a substituted anthraquinone having the formula

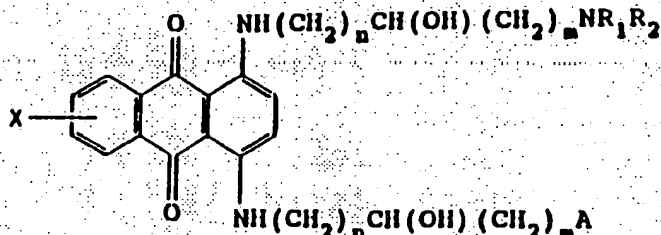


- 10 wherein  $R_1$  and  $R_2$  are independently H,  $C_1$ - $C_{10}$  alkyl, aryl, arylalkyl, alkylaryl, or  $R_1$  and  $R_2$  taken together with the adjacent nitrogen atom N form a substituted or unsubstituted cyclic ring which may contain additional heteroatoms; n and m are independently 1, 2, or 3; A is
- 15 Halogen, OH, alkoxy,  $\text{OCO}(\text{NR}^3\text{R}^4)$ ,  $\text{S}-\text{C}(\text{NH}_2)=\text{NR}_5$ , or when m = 1, comprises an oxirane ring with the adjacent oxygen atom;  $R_3$ ,  $R_4$ , and  $R_5$  are independently H, alkyl, or aryl; X is H, OH,  $\text{NR}_6\text{R}_7$ , Cl, Br, I, F, alkyl, aryl, alkoxy, aryloxy,  $\text{COOR}_8$ , or  $\text{CONR}_9\text{R}_{10}$ ; and  $R_6$ ,  $R_7$ ,  $R_8$ ,  $R_9$ , and  $R_{10}$  are
- 20 independently H, lower alkyl, or aryl.
8. The method of claim 7 wherein  $R_1$  and  $R_2$  independently are H or lower alkyl; n and m are independently 1 or 2; A is halogen,  $\text{OCO}(\text{NR}_3\text{R}_4)$ ,  $\text{SC}(\text{NH}_2)=\text{NR}_5$ , or when m = 1, comprises an oxirane ring with the adjacent oxygen atom;
- 25  $R_3$ ,  $R_4$ , and  $R_5$  are independently H or alkyl; and X is H, HO, Cl, Br, I, F, or alkoxy.
9. The method of claim 8 wherein  $R_1$  and  $R_2$  independently are lower alkyl; m and n are 1; A is Cl, Br,  $\text{OCO}(\text{NR}_3\text{R}_4)$ ,  $\text{SC}(\text{NH}_2)=\text{NR}_5$ , or when m = 1, comprises an oxirane ring with

- 35 -

the adjacent oxygen atom;  $R_3$ ,  $R_4$ , and  $R_5$  are H; and X is H or OH.

10. The use of a compound having the formula



5 wherein  $R_1$  and  $R_2$  are independently H,  $C_1$ - $C_{10}$  alkyl, aryl, arylalkyl, alkylaryl, or  $R_1$  and  $R_2$  taken together with the adjacent nitrogen atom N form a substituted or unsubstituted cyclic ring which may optionally contain additional heteroatoms; n and m are independently 1, 2, or  
 10 3; A is Halogen, OH, alkoxy,  $OCO(NR_3R_4)$ ,  $S-C(NH_2)=NR_5$ , or when  $m = 1$ , comprises an oxirane ring with the adjacent oxygen atom;  $R_3$ ,  $R_4$ , and  $R_5$  are independently H, alkyl, or aryl; X is H, OH,  $NR_6R_7$ , Cl, Br, I, F, alkyl, aryl, alkoxy, aryloxy,  $COOR_8$ , or  $CONR_9R_{10}$ ; and  $R_6$ ,  $R_7$ ,  $R_8$ ,  $R_9$ , and  $R_{10}$  are  
 15 independently H, lower alkyl, or aryl in the preparation of a medicament for inhibiting protein kinase C.

11. The use of claim 10 wherein said compound has the formula of claim 40 wherein  $R_1$  and  $R_2$  independently are H or lower alkyl; n and m are independently 1 or 2; A is  
 20 halogen,  $OCO(NR_3R_4)$ ,  $SC(NH_2)=NR_5$ , or when  $m = 1$ , comprises an oxirane ring with the adjacent oxygen atom;  $R_3$ ,  $R_4$ , and  $R_5$  are independently H or alkyl; and X is H, HO, Cl, Br, I, F, or alkoxy.

12. The use of claim 11 wherein said compound has the  
 25 formula of claim 40 wherein  $R_1$  and  $R_2$  independently are lower alkyl; m and n are 1; A is Cl, Br,  $OCO(NR_3R_4)$ ,  $SC(NH_2)=NR_5$ , or when  $m = 1$ , comprises an oxirane ring with the adjacent oxygen atom;  $R_3$ ,  $R_4$ , and  $R_5$  are H; and X is H or OH.

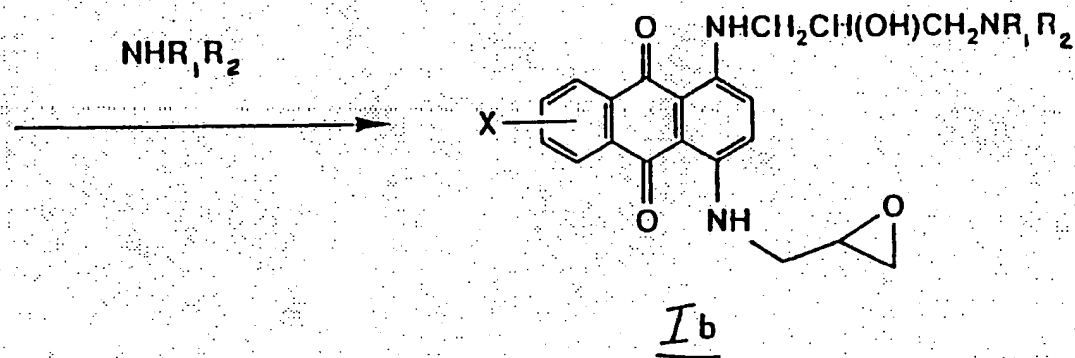
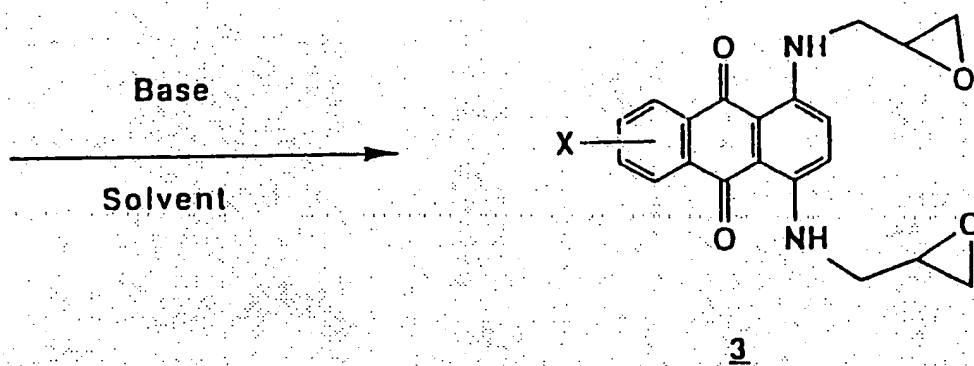
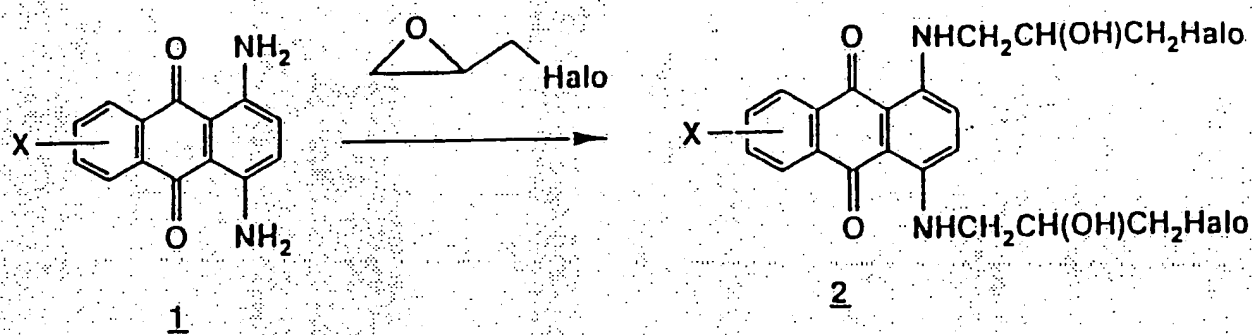
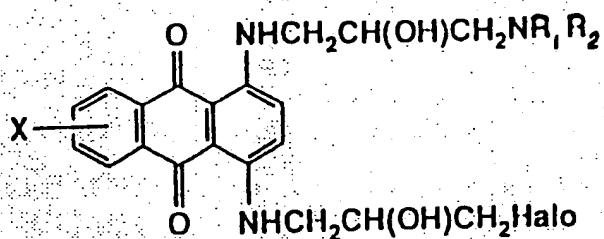
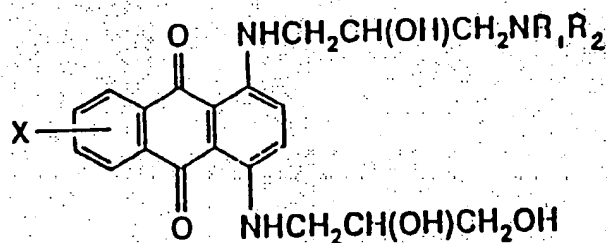


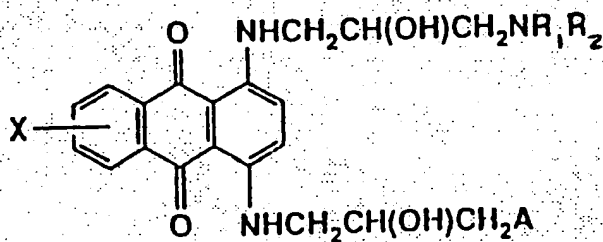
Figure 2



Ia



Ib

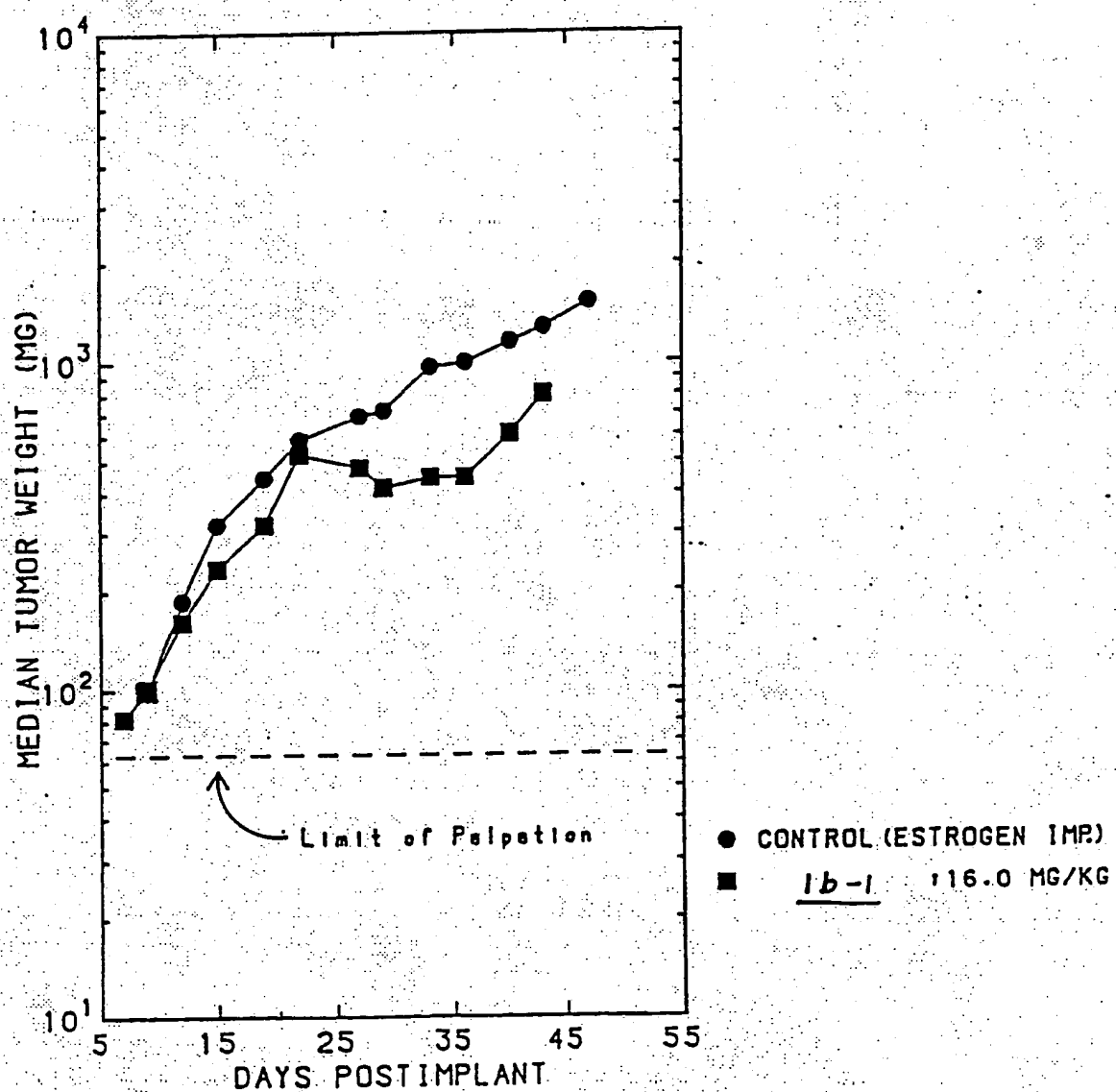


A = Alkoxy,  $\text{OCO}(\text{NR}_3\text{R}_4)$ ,  $\text{SC}(\text{NH}_2)=\text{NR}_5$

Figure 3



FIGURE 4



# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/08026

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC(5): A61K 31/045, 12, 085, 095, 335; C07D 303/08, 32, 34, 36, ; C07C 225/34		
U.S. 514/475, 508, 510, 514, 538, 561, 563, 564, 620, 621, 622, 656, 680; 549/551, 553; 552/236, 238, 240, 243, 249, 251, 255, 260		
<b>II. FIELDS SEARCHED</b> 243, 249, 251, 255, 260		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
U.S.	514/475, 508, 510, 514, 538, 561, 563, 564, 620, 621, 622, 656, 680; 549/551, 553; 552/236, 238, 240, 243, 249, 251, 255, 260	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
CAS ONLINE, THE FORMULA OF CLAIM 1		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT *</b>		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
A	US, A, 1,540,733 (BADDILEY ET AL.) 02 June 1925, See the entire document.	1-6
A	US, A, 4,762,648 (STACHE ET AL.) 09 August 1988, See the entire document.	1-6
A	US, A, 4,310,666 (ZEE-CHENG ET AL) 12 January 1986, See the entire document.	1-13
A	US, A, 4,816,450 (BELL ET AL.) 28 March 1989, See the entire document.	7-13
A	US, A, 4,894,451 (KRAPCHO ET AL.) 16 January 1990, See the entire document.	1-13
<p>* Special categories of cited documents: 10</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"d" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
05 MARCH 1992	26 MAR 1992	
International Searching Authority	Signature of Authorized Officer	
ISA/US	RAYMOND COVINGTON	

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_ because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claim numbers 1, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out<sup>12</sup>, specifically:

Claim 1 where R<sub>1</sub> and R<sub>2</sub> form a heterocyclic ring has been searched only to the extent of specifically disclosed or claimed species as the claim includes within its' scope inoperable species, such as, poly Te containing rings, 3 member rings having 2 nitrogens, or 8 member rings with poly adjacent oxygens.

3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VL ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.